Streptomyces clavuligerus: The Omics Era

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Abstract: The *Streptomyces clavuligerus* genome consists in a linear chromosome of about 6.7 Mb and four plasmids (pSCL1 to pSCL4), the latter one of 1.8 Mb. Deletion of pSCL4, results in viable mutants with high instability in the chromosome arms, which may lead to chromosome circularisation. Transcriptomic and proteomic studies comparing different mutants with the wild-type strain improved our knowledge on the biosynthesis and regulation of clavulanic acid, cephamycin C and holomycin. Additional knowledge has been obtained on the SARP-type CcaR activator and the network of connections with other regulators (Brp, AreB, AdpA, BldG, RelA) controlling *ccaR* expression. The transcriptional pattern of the cephamycin and clavulanic acid clusters is supported by the binding of CcaR to different promoters and confirmed that ClaR is a CcaR-dependent activator that controls the late steps of clavulanic biosynthesis. Metabolomic studies allowed the detection of new metabolites produced by *S. clavuligerus* such as naringenin, desferroxamines, several *N*-acyl tunicamycins, the terpenes carveol and cuminyl alcohol or bafilomycin J. Heterologous expression of *S. clavuligerus* terpene synthases resulted in the formation of no less than 15 different terpenes, although none of them was detected in *S. clavuligerus* culture broth. In summary, application of the Omic tools results in a better understanding of the molecular biology of *S. clavuligerus,* that allows the use of this strain as an industrial actinobacterial platform and helps to improve CA production.

Keywords: *Streptomyces clavuligerus,* Clavulanic acid, Cephamycin C, Clavams, Terpenes, Secondary metabolites

Streptomyces clavuligerus is the strain used industrially to produce clavulanic acid (CA). This compound is a potent β-lactamase inhibitor that binds the serine residue in the active centre of β-lactamases causing their irreversible inactivation and is used to potentiate the activity of classical β-lactam antibiotics against resistant bacteria. In addition, *S. clavuligerus* synthesises the β-lactam antibiotic cephamycin C and other compounds with antibacterial and antifungal activities. These facts made *S. clavuligerus* an interesting subject of study and numerous articles have been published on the physiology, nutrition, fermentation, and on enzymes and regulatory mechanisms involved in the formation of the metabolites produced by this species. The recent availability of powerful Omics tools provides new molecular genetic information on *S. clavuligerus*. The aim of this article is to analyse the impact of the Omics technology on the present knowledge on *S. clavuligerus,* especially in relation to secondary metabolite biosynthesis. The annotation of *S. clavuligerus* genes cited in this work is presented as in the *Streptomyces* database, StrepDB, and the equivalence to NCBI annotations is shown in Table S1.

Genomic of *Streptomyces clavuligerus*

S. clavuligerus, as all *Streptomyces* species, possess a linear chromosome. In addition, Netolitzky et al. (1995) using pulsed-field gel electrophoresis, found in the type strain *S. clavuligerus* NRRL 3585 three plasmids named pSCL1 (11.7 kb), pSCL2 (120 kb) and pSCL3 (430 kb). This research group also sequenced pSCL1 and partially pSCL2 (Wu & Roy, 1993; Wu et al., 2006).

The *S. clavuligerus* genome has been sequenced and analysed by several research groups. The Broad Institute (2008) was first to make public a very fragmented unannotated assembly of *S.* *clavuligerus* ATCC 27064 genome (Table 1). Medema et al. (2010) using Sanger sequencing of whole-genome shotgun libraries published the first *S. clavuligerus* ATCC 27064 genome assembly consisting of single scaffolds per replicon, and showed for first time the presence of a megaplasmid of 1,796 kb, named pSCL4, together with a chromosome of 6,760 kb (Table 1). The sequences described for pSCL2 were not found by these authors neither replicons of the sizes of pSCL2 and pSCL3. In parallel, Song et al. (2010) using a Sanger paired-end sequencing and 454 pyrosequencing published the *S. clavuligerus* NRRL 3585 genome (NRRL 3585 and ATCC 27064 refer to the type strain deposited in different culture collections) comprised by a 6.7-Mb chromosome and four linear replicons corresponding by size to the pSCL1, pSCL2, pSCL3 and pSCL4 plasmids. A refined genome of *S. clavuligerus* ATCC 27064 was obtained using a PacBio genome library and Illumina sequencing (Hwang et al., 2019). These authors published an assembly with single contigs for the chromosome (6.75 Mb) and pSCL4 (1.8 Mb), and annotated 7,163 genes including 6,880 protein-encoding genes, 196 pseudogenes, 66 tRNAs, 18 rRNAs and 3 ncRNAs; this assembly however does not contain any data on any of the other three plasmids. An additional genome assembly (GCA_015708605.1) has been recently made public from an *S. clavuligerus* ATCC 27064 isolate studied for more than 25 years in the Microbiology laboratory at the University of León in Spain (Gomez-Escribano et al., 2021). This is a refined sequence (200× PacBio coverage and optical mapping) in which plasmids pSCL1, pSCL2 and pSCL4 were sequenced close to completion but plasmid pSCL3 was not found. The authors, intrigued by the presence of pSCL3 only in the NRRL 3585 genome assembly from Song and colleagues (2010), further assessed the presence of plasmids in five different genuine type strain in culture collection by Illumina deep whole-genome sequencing, and found all four plasmids in

Table 1. *Streptomyces clavuligerus* Chromosome and Plasmid Sequences Available at NCBI

Strain	Method used (Coverage)	Replicons	Size (bp)	CG (%)	Gene number	Reference and accesion number
S. clavuligerus NRRL 3585	Sanger	Isolated plasmid pSCL1	11,696			Wu and Roy, 1993 X54107
S. clavuligerus NRRL 3585	Sanger	Isolated plasmid pSCL2	Partial sequence			Wu et al., 2006 AY392418
S. clavuligerus ATCC 27064	Unknown	597 ungapped contigs (6,729,086 bp)	6,941,740			Broad Institute, 2008 GCA_000154925
S. clavuligerus ATCC 27064	Sanger (11,8×)	Chromosome plasmid pSCL4	6,760,392 1,799,650		7281	Medema et al., 2010 ADGD01000000
S. clavuligerus NRRL 3585	Sanger/454 pyrosequencing (80,7×)	Chromosome Plasmid pSCL1 Plasmid pSCL2 Plasmid pSCL3 Plasmid pSCL4	6,736,473 10,266 149,326 442,792 1,796,117	72.7 71.9 70.0 70.7 71.8	7655	Song et al., 2010 ADWJ01000000
S. clavuligerus F613-1	PacBio RSII/Illumina (60×)	Chromosome Plasmid pSCL4-like	6,883,702 707,056	72.68 71.82	6340	Cao et al., 2016 CP016559/CP016560
S. clavuligerus F1D-5	PacBio/Illumina (320×)	Chromosome Plasmid pSCL1 Plasmid pSCL2	6,900,908 1,051,520 106,697		6692	Shandong Academy of Medical Science, 2018 GCA_003454 755
S. clavuligerus ATCC 27064	PacBio/Ilumina (50×)	Chromosome-Plasmid pClaA1	6,784,591 1,795,495		7163	Hwang et al., 2019 GSE128216
S. clavuligerus ATCC 27064, León strain	PacBio/Ilumina (200×)	Chromosome Plasmid pSCL1 Plasmid pSCL2 Plasmid pSCL4	6,748,580 11,696 149,702 1,794,824		7227	Gomez-Escribano et al., 2021 GCA_015708605

all type strain deposits; this finding prompted the authors to suggest that pSCL3 might have been lost in the León isolate during subculturing of the original ATCC 27064 culture (Gomez-Escribano et al., 2021). Finally, the genomes of two industrial strains sequenced with PacBio/Ilumina have been described: *S. clavuligerus* F613-1 from which was reported a chromosome of 6.88 Mb and one plasmid of 0.7 Mb with sequence partially identical to pSCL4 (Cao et al., 2016) and *S. clavuligerus* F1D-5. The results obtained from these studies are summarised in Table 1.

As usual in other *Streptomyces* species, *S. clavuligerus* linear chromosome has a conserved core region, rich in primary metabolism and housekeeping genes, and two chromosomal arms in which many secondary metabolites encoding clusters are located. The chromosome size is smaller than the average *Streptomyces* chromosomes probably due to the large 1.8-Mb pSCL4 plasmid. The differences in chromosome size and the number of plasmids found by different authors suggest that *S. clavuligerus* has a very dynamic genome due to plasmid loss and events of plasmid/chromosome recombination. In industrial strains the instability increases due to the effect of heavy mutagenesis as shown by the pSCL4 behaviour in strain *S. clavuligerus* F613 (Cao et al., 2016).

Plasmid pSCL4 does not have housekeeping genes except for some tRNA and rRNA genes which are duplicated in the chromosome but carries numerous gene clusters for secondary metabolites. A metabolic model for the growth of *S. clavuligerus* on several carbon and nitrogen sources, predicted *in silico* that removal of pSCL4 will not affect essentially the strain growth suggesting that this plasmid was not essential (Medema et al., 2010). This was experimentally confirmed by quantification of pSCL4 copy number in cultures of strain *S. clavuligerus oppA2::aph*, a strain that has about 25,000-fold lower pSCL4 copy number in relation to total

DNA, than cultures of the parental strain (Álvarez-Álvarez et al., 2014a). A similar mechanistically unpredictable loss of pSCL4 was reported by Charusanti et al. (2012) due to selective pressure by co-culturing *S. clavuligerus* with *Staphylococcus aureus*.

Removal of pSCL4 by Deletion of the *par* Genes: Effect on Expression of Chromosomal Genes

A pSCL4-free strain, *S. clavuligerus* pSCL4⁻, was obtained by deletion of the pSCL4 *parA-parB* genes which differ 60–70% in sequence from the *par* genes required for chromosomal segregation. The pSCL4-free mutant fails to sporulate, grows more slowly than the parental strain and produced 20–30% less clavulanic acid. Colonies of the pSCL4-less strain have a spectacular yellow colour due to overproduction of the antibiotic holomycin, whose biosynthesis is deregulated in these mutants (Álvarez-Álvarez et al., 2014a, 2017). Comparative genomic and transcriptomic studies between the wild-type and the pSCL4-deleted strains reveal a high instability of *S. clavuligerus* genome. q-PCR quantification demonstrates that the lack of expression of genes in a 303-kb DNA fragment of *S. clavuligerus* pSCL4⁻ chromosome right arm, was due to the absence of these genes. The chromosomal 303-kb DNA fragment, including the right telomere, probably was translocated to pSCL4, and lost in the plasmid-less mutant.

Recently CRISPR-cas9-mediated targeting of pSCL4 *parB* gene resulted in new pSCL4-free strains (Gomez-Escribano et al., 2021). Five pSCL4-cured isolates were sequenced and all of them showed deletions in one or both chromosomal arms, and other rearrangements, although they kept plasmids pSCL1 and pSCL2. For two of the mutants enough sequence data were obtained to show circularisation of the chromosome concomitant to the loss of around 100 kb of each end. The deletions are not site specific

as they range from 88 kb to 303 kb (Álvarez-Álvarez et al., 2017; Gomez-Escribano et al., 2021) in the right arm of the chromosome, but probably chromosome circularisation relies on the deletion of fragments at both arm ends (Chen et al., 2002; Tidjani et al., 2020). From these results it can be concluded that pSCL4 is not essential for *S. clavuligerus* viability since the pSCL4-less mutant growth is only slightly delayed in relation to the wild-type strain; however, pSCL4 appears to be essential for the stability and maintenance of the linear chromosome, a genetic hallmark of streptomycetes, and it is still to be elucidated whether the lack of morphological development of pSCL4-cured strains is due to the loss of specific genes located in pSCL4 or the loss of chromosomal sequence.

Alvarez-Alvarez and colleagues found that deletion of pSCL4 resulted in a strong effect on the transcription of many chromosomal genes including genes for several regulators. The pSCL4-less strain upregulates the defence system for oxidative stress, the signal transduction system mediated by *sigE-cseABC* and downregulates the survival mechanisms mediated by the *mce* cluster (SCLAV_5177/_5193) (Álvarez-Álvarez et al., 2017). In addition, expression of genes for holomycin, tunicamycin and a type I polyketide compound (SCLAV_0446/_0497) biosynthesis were upregulated. Other gene clusters, as those for clavulanic acid, cephamycin C (SCLAV_4179/4217), desferrioxamine (SCLAV_1948/_1951), a lantibiotic-type compound (SCLAV_4387/_4392), an NRPS-type compound (SCLAV_5608/_5638) and for terpene biosynthesis (SCLAV_5670/_5674) were downregulated. Metabolomic studies confirmed that the products formed by *S. clavuligerus* pSCL4$^-$ differ significantly from those of the wild-type strain (see below). The requirement of a correct pSCL4 copy number for optimal clavulanic acid production is an important contribution of the genomic studies.

Transcriptomic and Proteomic Studies

Transcriptomic and proteomic studies in *Streptomyces* have been made by different authors using distinct strains and conditions: (a) studying the sequential expression of genes at different times of growth (Botas et al., 2018; Fergusson et al., 2016); (b) comparing gene expression in different culture media (Hwang et al., 2019; Pinilla et al., 2019); (c) studying the wild-type strain in relation to particular mutants (Álvarez-Álvarez et al., 2014b, 2017, 2018; Martínez-Burgo et al., 2015, 2019; Pérez-Redondo et al., 2012), and (d) comparing the wild-type strain with industrial strains (Medema et al., 2011; Ünsaldi et al., 2017). Transcriptomic studies provide a wealth of information that frequently is difficult to interpret and will require years of research to understand the up- and downexpression of hundreds of genes reported in the different studies. However, the differences in regulation of whole-gene clusters might be understood with the present available knowledge. In this article we will focus mostly on the transcriptomic studies of gene cluster expression, particularly of the clusters encoding secondary metabolites.

The genomic positions of transcription start sites (TSSs) have been determined in the complete *S. clavuligerus* genome, at four growth phases of the culture using an RNAseq library (Hwang et al., 2019). These authors found A/G-rich TSS in most *S. clavuligerus* genes. The −10 sites sequence, TANNNT, is conserved in about 90% of *S. clavuligerus* promoters and separated by an average distance of 18–19 nt from the less conserved, NTGAC, −35 sequence. The more abundant Shine–Dalgarno sequence in *S. clavuligerus* is RRGGAG. The *S. clavuligerus* regulons were proposed to be identified regarding their −35 promoter sequences (Hwang et al., 2019), which are recognised by specific sigma factors (Chauhan et al., 2016). Following this hypothesis Hwang et al. assumed that the SigF-binding RNA polymerase transcribes *argR,* and the ArgR regulator controls the arginine biosynthesis genes and the formation of clavulanic acid. However, experimental studies are required to confirm this claim. In a different study, these authors determined the 3′-end position (TEP) of 1,427 *S. clavuligerus* transcripts using a Term-seq library and classified the RNAs according to the free energy of the secondary structure of the TEP regions and the uridine chain downstream of the stem structure (Hwang et al., 2021). Specifically, the TSS and TEP structures of the 58 secondary metabolite gene clusters were studied and the transcripts of the CA and cephamycin gene clusters were confirmed.

The most complete *S. clavuligerus* proteome analysis reported 2,442 proteins, about 33% of the proteome, and compared them in the wild-type strain and two mutants deleted, respectively, in the regulatory genes *bldA* and *bldG* (Ferguson et al., 2016). The *bldA* gene encodes a leucine-specific tRNA that translates the rare TTA codon (Chandra & Chater, 2008) while *bldG* encodes an anti-anti-sigma factor (see below). Proteomic studies showed that the BglG factor is strongly downrepresented in the *bldA* mutant (Fergurson et al., 2016). Ünsaldi et al. (2017) compared 93 proteins in *S. clavuligerus* NRRL 3585 and the industrial clavulanic producer strain *S. clavuligerus* DEA. They found that proteins involved in CA formation (CeaS2, Bls2, Car) were overrepresented in the industrial strain but surprisingly the regulators CcaR, ClaR and the putative CagR regulator, were not. Partial proteome studies have been made also comparing *S. clavuligerus* ATCC 27064 and mutants in the *ccaR, claR, oppA2* or *argR* genes (Nárdiz et al., 2011; Robles Reglero et al., 2013) and in the *hom* gene, encoding the homoserine dehydrogenase (Ünsaldi et al., 2021) as a way to understand the role of these genes in clavulanic acid and cephamycin C production.

An interesting fact observed in proteomic studies is the presence of multiple spots corresponding to the same protein. Sixteen proteins appeared in two or more spots in the two-dimensional gels reported by Ünsaldi et al. (2017) suggesting post-translational modifications. As examples, the cystathionine-γ-synthase (SCLAV_5668) is represented by five spots and MetE by two isoforms. Also, the carboxyethylarginine synthetase (CeaS2) and the catalase of *S. clavuligerus* appear in several spots (Lorenzana & Liras, unpublished results). Although of interest, large-scale proteomic studies frequently are difficult to interpret and require parallel transcriptomic and physiological studies.

Organisation of the Genes of Cephamycin C, Clavulanic Acid and Clavams in *Streptomyces clavuligerus* Genome

Using AntiSMASH 4.0, Hwang et al. (2019) predicted that *S. clavuligerus* genome contains 58 gene clusters for secondary metabolites, 30 of them located in the chromosome and 28 in the megaplasmid pSCL4. This indicates that 21.7% of the genes in the genome are involved in secondary metabolite formation. A small number of these clusters have been thoroughly studied from the biochemical and genetical point of views. They are the clusters for the β-lactams (clavulanic acid, cephamycin C, clavams), for holomycin and for tunicamycin that will be considered in next section. In recent years additional metabolites produced by *S.*

clavuligerus have been detected using mass spectrometry (MS) and nuclear magnetic resonance (NMR). These compounds and the possible clusters encoding them will be considered in a later section.

The Clavulanic Acid Pathway and Gene Cluster

From the pharmacological and industrial point of view, the most important compound produced by *S. clavuligerus* is clavulanic acid. The chemistry, biosynthetic pathway, enzymes and regulation of clavulanic acid formation have been reviewed previously (Baggaley et al., 1997; Hamed et al., 2013; Jensen & Paradkar, 1999; Liras & Rodríguez-García, 2000; Liras et al., 2008, 2011; Townsend, 2002). In addition, other reviews deal with the industrial production of clavulanic acid and its purification (López-Agudelo et al., 2021; Saudagar et al., 2008) or with strategies to obtain CA high-producing strains (Paradkar, 2013). Therefore, this article will focus only on the recent results on clavulanic acid biosynthesis and regulation, obtained mostly using Omics technology during the past 15 years.

Cephamycin C, clavulanic and clavams are β-lactam antibiotics, containing a four-membered β-lactam ring. Moreover, the cephamycin C molecule has a six-membered dihydrothiazinic ring containing sulphur while clavulanic acid and the clavams possess a five-membered clavam ring containing oxygen. Although the initial steps of clavams and CA pathways are common, the stereochemical configuration of clavulanic acid is (3R, 5R) while the antifungal clavams have a (3S, 5S) stereochemistry and lack β-lactamase inhibitory properties. The biosynthetic pathway leading to cephamycin C is unrelated to that of CA; however, the cephamycin C and CA gene clusters in *S. clavuligerus* are adjacent forming a 60-kb supercluster (Fig. S1A). Expression of the genes in this supercluster is initiated by a common activator protein named CcaR encoded by *ccaR, a* gene located in the cephamycin C gene cluster. A second regulatory gene *claR*, located in the CA cluster encodes ClaR, an activator that controls specifically the late steps of clavulanic acid biosynthesis but not the formation of cephamycin C or clavams.

A similar supercluster organisation occurs in *Streptomyces jumonjinensis* and *Streptomyces katsurahamanus* (AbuSara et al., 2019; Nobary & Jensen, 2012; Ward & Hodgson, 1993) with small modifications in the order of some genes and the lack of genes *blp,* for a putative protein inhibitory of β-lactamases, and *pcbR,* confering β-lactam resistance. The three *Streptomyces* species produce equivalent amounts of cephamycin C, but clavulanic acid production in the *S. clavuligerus* wild type is about five- to sixfolds higher than in the other species. Other *Streptomyces* species contain only the genes for clavulanic acid production; the best studied are *Streptomyces flavogriseus* ATCC 33331 (renamed *Streptomyces pratensis*) and *Saccharomonospora viridis* DSM 43017 (Álvarez-Álvarez et al., 2013; Nobary & Jensen, 2012). These *Streptomyces* species have a silent CA cluster and, at difference of *S. clavuligerus,* carry the *ccaR* gene in the CA cluster. RT-PCR studies of *S. flavogriseus* show that five essential genes for CA biosynthesis are not expressed in this strain, even when the heterologous *ccaR* gene of *S. clavuligerus,* was expressed in *S. flavogriseus*. Probably S. *flavogriseus* has a poor conservation of nucleotides in the CcaR-binding sequences, since the *S. flavogriseus* CcaR protein is functional and able to activate the CA gene cluster of *S. clavuligerus* (Álvarez-Álvarez et al., 2013).

Clavulanic acid biosynthesis: New insights into genes and proteins

S. clavuligerus is a peculiar strain that does not use glucose because it lacks expression of the glucose permease gene, *glP1*

(Pérez-Redondo et al., 2010), however, it uses efficiently glycerol and amino acids as carbon sources. CA is formed from two precursors, glyceraldehyde-3-phosphate and arginine (Fig. S1B). Improved CA-producing strains have been obtained by increasing the glyceraldehyde-3-P pool through genetic engineering of the glycolytic pathway (Li & Townsend, 2006), by increasing the copy number of the *glpK1D1* glycerol biosynthesis genes and by addition of glycerol to the cultures (Baños et al., 2009; Shin et al., 2021). The gene cluster for arginine and the arginine gene cluster regulation in Streptomyces have been thoroughly studied using genetic and transcriptomic tools and this might be useful to improve CA production (Botas et al., 2018; Pérez-Redondo et al., 2012; Rodríguez-García et al., 2000). The CA pathway starts with the condensation of glyceraldehyde-3-P and arginine by the carboxyethylarginine synthase (CeaS2), formation of a β-lactam ring by the β-lactam synthetase (Bls2), closing of the clavam ring by the multifunctional clavaminate synthase (Cas2), able to hydroxylate, cyclisise and desaturate its substrate in a single-binding cavity leading to the formation of (3S, 5S) clavaminic acid (Wojdyla & Borowski, 2021). This compound is the start point of a lateral branch of the CA pathway that results in the formation of (3S, 5S)-clavams (see below).

In the late steps of the CA pathway clavaminic acid is converted to (3S, 5S) *N*-glycylclavaminic acid by an ATP grass fold family protein (GcaS) that utilises glycine and ATP to introduce a glycyl group at the C9 amino group of clavaminic acid. Further steps in CA are poorly understood. However, mutations of several genes (*oppA1, oppA2, cyp, orf12, 13, 14, 16*) located in the CA cluster indicate that they are essential for CA formation suggesting the involvement of several additional enzymatic steps in the pathway, including the key change of (3S, 5S) to (3R, 5R) stereochemistry present in CA, which is essential for its β-lactamase inhibitory activity.

It was known that mutants deleted in *oppA2*, encoding an oligopeptide-binding protein, release large amounts of *N*-acetylglycylclavaminic acid (NAGC) to the broth (Jensen et al., 2004; Lorenzana et al., 2004). Formation of NAGC from *N*-glycylclavaminic acid requires an acetylation step, since the GcaS enzyme does not use *N*-acetylglycine as substrate (Arulanantham et al., 2006). Crystallised OppA2 protein is able to bind dipeptides or tripeptides containing arginine (MacKenzie et al., 2010); therefore, it was hypothesised that OppA2 could also bind NAGC, what might explain the release of NAGC by mutants lacking the OppA2 protein. Surprisingly, the co-culture of a CA non-producer *oppA2* mutant with CA non-producer mutants blocked in genes for the early steps of the pathway (*ceaS2* or *bls2*), results in CA formation by the mixed culture. This co-synthesis of CA does not occur when the *oppA2*-deleted mutant is co-cultured with mutants blocked in the *cyp*, *orf14* or *oppA1* genes, putatively involved in late steps of the pathway. Formation of CA is also observed in solid and liquid cultures when sterile broth of *oppA2*-null cultures, or pure *N*-acetylglycylclavaminic, is added to the cultures of *ceaS2*- or *bls2*-mutants, confirming that *N*-acetylglycylclavaminic is a real intermediate in the CA biosynthesis pathway (Álvarez-Álvarez et al., 2018). Transcriptomic studies show that only 233 genes changed their expression due to the lack of OppA2. They include genes for proteases and transport systems, but expression of all the CA biosynthesis genes was not significantly affected. This important advance in the knowledge of the CA pathway suggests that NAGC is formed by acetylation of *N*-glycylclavaminic acid. The release of *N*-acetylglycylclavaminic to the medium indicates that OppA2 binds this compound at the membrane level and presents it to the next enzyme in the pathway.

Expression of six additional genes in the CA gene cluster (*oppA1, cyp-fd, orf12, orf13, orf14, orf16*) is still required for CA production.

No additional advances have been made on the mechanisms of action of the products of the genes: (1) *cyp-fd*, encoding a P450-ferredoxin complex (Li et al., 2000), (2) *orf13*, encoding a protein of the Eam family similar to the *O*-acetylserine/cysteine exporters, and (3) *orf14/orf16* both of them encoding proteins with the GNAT motif of acetyltransferases. However, the proteins encoded by *orf12* and *orf14* have been crystallised. Protein Orf12 resembles type A β-lactamases and has a β-lactamase-like domain at the C-terminal end, although lacks *in vitro* β-lactamase activity (Valegard et al., 2013). Orf12 does not have amidase activity or the DD-carboxypeptidase activity characteristic of PBP proteins. Pure ORF12 is able to hydrolyse with low efficiency the 3′-O-acetyl side chain of cephalosporin C and also catalyses the reverse reaction, that is, the acetylation of deacetylcephalosporin C; therefore, this enzyme has been named Cpe for cephalosporin esterase (Srivastava et al., 2019). Disruption of the *cpe* gene does not affect cephamycin C production but completely blocks clavulanic acid formation although all the genes of the CA cluster are transcribed in this mutant. Although is a nucleophilic hydrolase, the Cpe protein lacks detectable peptide signal for secretion and Western blotting confirms that is a cytoplasmic protein suggesting that the cephalosporin C esterase/acetylase activities are not its real function. Pure Cpe protein has at the N-terminal end a domain isomerase/cyclase, characteristic of steroid isomerases, and is able to bind non-covalently clavulanic acid. For these reasons it has been suggested that Cpe may be involved in the opening of the clavam ring and its isomerisation to the 3R, 5R stereochemistry (Scrivastava et al., 2019). The isomerisation reaction has been also studied through a computational assisted approach (Gómez et al., 2020).

Orf14 possesses in the N- and C-terminal ends two motifs characteristic of the tandem GNAT-superfamily acetyl transferases. The crystal structure of Orf14 has an acetyl-CoA molecule deeply buried in the N-terminal domain, suggesting that this acetyl-CoA molecule has a structural rather than catalytic role. A second acetyl-CoA is bound by the protein, probably at the C-terminal domain (Iqbal et al., 2010). These authors propose that the C-terminal end is the catalytic site of the protein that may use acetyl-CoA or other acylated-CoA substrates. However, the involvement of Orf14 in *N*-acylglycylclavaminic acid formation has not been reported.

The final reaction of the CA pathway involves the action of the clavaldehyde dehydrogenase, Car, a tetrameric protein that uses NADPH to reduce the 3R, 5R clavaldehyde to 3R, 5R clavulanic acid (Fig. S1B).

RT-PCR analysis of the CA gene cluster polycistronic transcripts reveals a major transcript of the genes for the early steps of the pathway (*ceaS2-bls2-pah2-cas2*), large transcripts of genes for the late steps of the pathway (*cyp-orf12-orf13* and *orf16-oppA2*) and genes transcribed individually (*claR, car, orf14, gcaS*) or transcribed both individually and forming part of larger transcripts (*cas2, oppA1, cyp, orf12, orf13*) (Hwang et al., 2021; Santamarta et al., 2011) (Fig. 1A).

The Antifungal Clavams Biosynthesis Pathway: Gene Clusters and Regulation

Clavams are metabolites with a (3S, 5S) five-membered clavam ring containing oxygen (Fig. S2). They have antifungal activity but lack β-lactamase inhibitory properties. Two gene clusters for clavam biosynthesis are present in *S. clavuligerus*, the clavam gene cluster (*cvm7-3-2-2-cas1-cvm4-5-6*; SCLAV_2922 to

_2929) and the CA paralogue gene cluster (*orfDCBA-ceaS1-bls1-pah1-oat1-cvm6p-7p-snk-res1-res2*; SCLAV_p1070 to _p1082), the latter located in plasmid pSCL4 (Tahlan et al., 2004, 2007). Studies using deleted mutants, elucidation of accumulated/or missing products of these strains, and comparison with the clavam clusters of other *Streptomyces* species, allowed to interpret the clavam biosynthetic pathway (Jensen, 2012; Tahlan et al., 2007). This pathway is branched and leads to the formation of alanylclavam and clavam-2-carboxylic acid, respectively; other previously reported clavams (2-formyloxymethylclavam, 2-hydroxymethylclavam) are intermediates in the pathway (Fig. S2). However, enzymatic studies to confirm the different steps and further research on the genes involved are still required. The *snk-res1-res2-cvm7P* genes, in the CA paralogue gene cluster, encode the clavam regulatory system (Kwong et al., 2012). Snk is a sensor kinase that is autophosphorylated and transfers the phosphate group to the two-component system Res1-Res2, in which Res1 is a truncated sensor and Res2 the response regulator. As detected by RT-PCR, deletion of *snk* and *res2* results in lack of transcription of several essential genes for clavam biosynthesis, including *cvm7p* for a SARP-LAL-type regulator. Mutations of *snk* and *res2* are complemented by transformation with the *cvm7p* parental allele expressed from a different promoter. In summary, Snk and the system Res1/Res2 control *cvm7p* transcription; in turn, Cvm7p activates expression of the clavam biosynthesis genes. The clavams and CA paralogue gene clusters are upregulated (1.5-fold) in a *ccaR* overexpressing, industrial strain (Medema et al., 2011); conversely, most of the genes in both clusters were downregulated in an *S. clavuligerus ccaR*-deleted strain (Álvarez-Álvarez et al., 2014b). The clavam activator gene *cvm7p* was weakly downregulated in this mutant while genes *cas1* and *orf8* were strongly upregulated suggesting that additional regulatory mechanisms control these clusters of genes (Álvarez-Álvarez et al., 2014b).

While the clavam gene clusters carry duplicated genes (*cas2, ceaS2, bls2, pah2* and *oat2*) and might increase CA production, clavams utilise part of the valuable CA precursors and interfere with CA purification; moreover, some clavams, as clavam-2-carboxylate, have toxic properties. Therefore, the lack of production of clavams is an advantage in CA improved strains (Paradkar, 2013).

Cephamycin C Biosynthesis and Gene Cluster

The cephamycin C gene cluster (SCLAV_4198 to _4217) contains (a) genes (*lat, pcd*) to form the cephamycin precursor L-α-aminoadipic acid, (b) genes for biosynthetic enzymes leading to deacetylcephalosporin C formation (*pcbAB, pcbC, cefD, cefE, cefF*), and (c) genes for the late modifications of deacetylcephalosporin C to form cephamycin C (*cmcI, cmcJ, cmcH*) (Fig. S1C). In addition, some genes tentatively involved in transport (*cmcT*) and antibiotic resistance (*bla, pcbR, pbp74*) are located in the cephamycin gene cluster. The most interesting gene in this cluster is *ccaR*, located downstream of *cmcH*, which encodes the SARP-type activator that controls expression of the cephamycin C and clavulanic acid biosynthesis genes. The cephamycin C biosynthesis pathway, the pathway intermediates (Fig. S1C) and the mode of action of the enzymes involved have been extensively studied and reviewed (Liras, 1999; Liras & Demain, 2009) and will not be considered here.

One of the enzymes involved in L-α-aminoadipic acid formation is the piperideine-6-carboxylate dehydrogenase (P6C-DH), which belongs to the aldehyde dehydrogenase superfamily. This enzyme was purified to homogeneity and characterised years ago
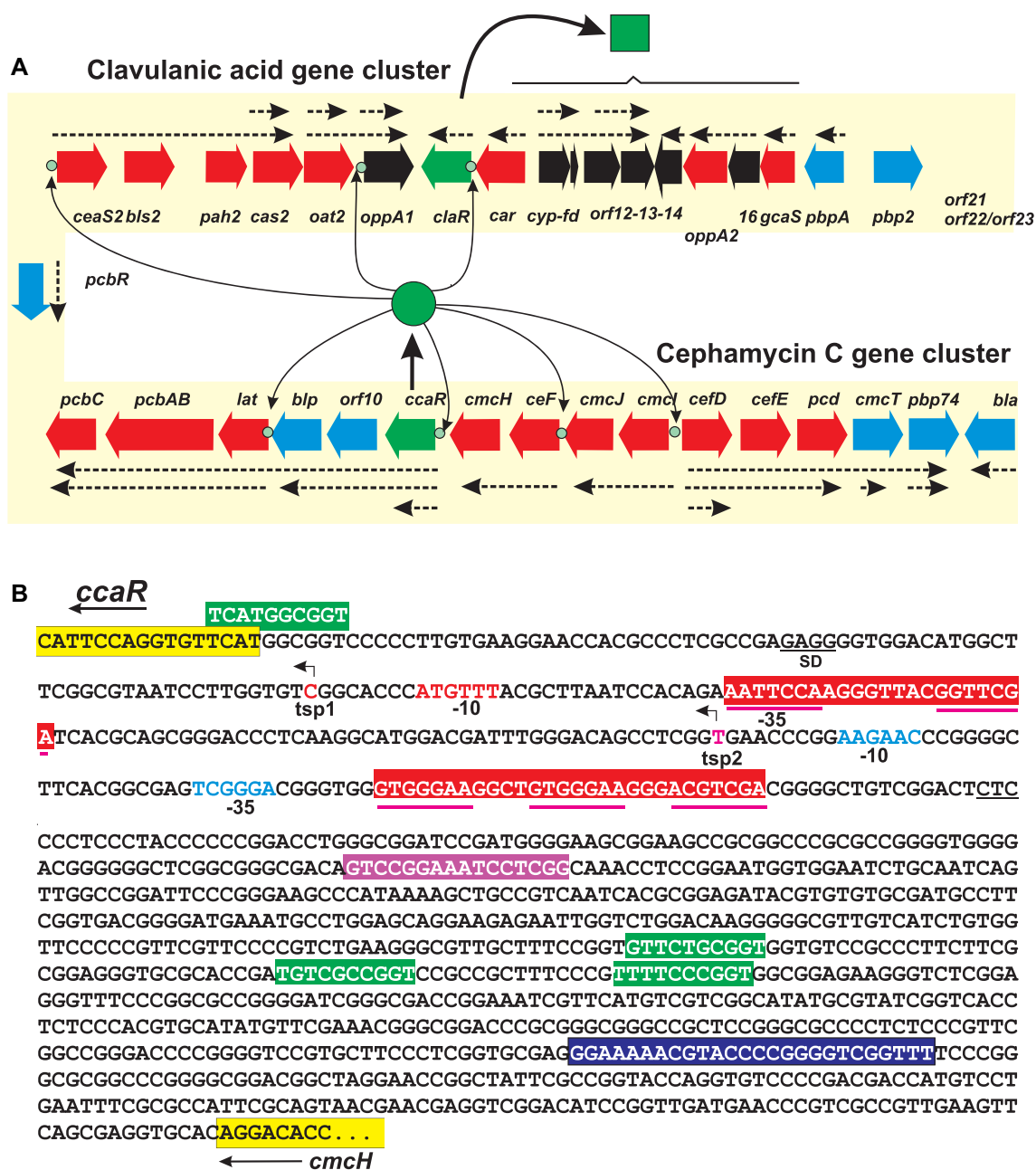
Fig. 1. Clusters of genes for clavulanic acid and cephamycin C biosynthesis and regulatory sites in the intergenic *ccaR-cmcH* region. (A) Cluster of genes for clavulanic acid biosynthesis (above) and for cephamycin C biosynthesis (below). The name of each gene is indicated. Notice that both clusters are linked through the *pcbR* gene (left side). Structural genes are shown in red colour and regulatory genes in green; genes for essential unknown functions are in black colour; genes for putative antibiotic resistance or transport are shown in blue. The known transcriptional units are indicated by dotted arrows. CcaR (green circle) binding sites are indicated as well as the putative sites for ClaR (green square) binding. (B) The sequence of the intergenic *ccaR-cmcH* is shown with the ends of the *ccaR* and *cmcH* genes boxed in yellow. The –35 site, –10 site, and the transcription start points are indicated with red lettering for *tsp1* and with blue lettering for *tsp2*. Four putative Adp-binding sites are boxed in green. The double and tripled heptameric sequences for CcaR binding are boxed in red. The sequence for ARE binding (ARE_ccaR) is boxed in dark blue. The putative sequence for PimM binding is shown in a purple box.

(Fuente et al., 1997) and has been recently crystallised (Hasse et al., 2019). Crystallographic studies indicated that this protein is a tetramer formed by a dimer-of-dimers (Hasse et al., 2019). Also, the methyl transferase CmcI involved in the C-7 methoxylation of the cephamycin nucleus has been crystallised (Öster et al., 2006). CmcI is a hexameric protein with a Rossmann domain at the C-terminal end that binds S-adenosylmethionine (SAM). The methyl group of SAM is transferred to the 7-α-OH cephalosporin in the final steps of formation of cephamycin C.

Expression of genes for cephamycin C biosynthesis proceeds through large transcripts expressing genes of the early-step enzymes (*lat, pacAB, pcbC*), the middle-step enzymes (*cefD, cefE, pcd, cmcT, pbpA*) and for enzymes acting late in the pathway (*cmcI, cmJ, cefF, cmcH*) (Fig. 1A). Some genes are transcribed individually or as bicistrons (*pcbC, cefD-cefE, bla, pcbR*). Finally, *ccaR* is expressed as a monocistronic unit and also coupled to *orf10* and *blp* (Hwang et al., 2021; Santamarta et al., 2011). This pattern of transcription is important to understand the regulation of the gene cluster.

Due to its industrial interest the whole *S. clavuligerus* cephamycin C gene cluster has been transferred to *Streptomyces coelicolor*, *Streptomyces albus* and *S. flavogriseus*. Only the last strain is able to express, at a low level, the cephamycin biosynthetic genes. However, even under optimal conditions, the heterologous cephamycin C production, confirmed by LC-MS, was only 8% of that of *S. clavuligerus* ATCC 27064. The genes *pcbA* and *pcbR* for β-lactam resistance were expressed at almost the same levels as in *S. clavuligerus,* and transformants of the three *Streptomyces* strains showed an increased resistance to penicillin and cefoxitin (Martínez-Burgo et al., 2014). These results point out to an important limitation of cephamycin overproduction in heterologous *Streptomyces* species probably due to the difficulty in duplicating the regulatory network of *S. clavuligerus* in heterologous systems.

Regulation of Clavulanic Acid and Cephamycin C Biosynthesis: the CcaR and ClaR Activator Proteins

As indicated above CcaR is a SARP-type activator that controls cephamycin C and clavulanic acid gene cluster expression. Transcription of *ccaR* starts from two different TSSs: a major transcription site, *tsp1,* located 75-nt upstream of the translation ATG codon and a *tsp2* site located at 173 nt (Wang et al., 2004). These *tsp* sites define two 5'-untranslated regions (UTR) that fit well with the average size of moderate and long UTRs described in *S. clavuligerus* (Hwang et al., 2019). RT-PCR studies show that a minor amount of *ccaR* transcript originates from the promoter of *cmcH* located upstream of *ccaR* (Kurt et al., 2013).

A second clavulanic acid regulatory gene, *claR,* is located in the CA cluster, downstream of *car,* encoding the clavaldehyde reductase. ClaR is a LysR-type regulator (Paradkar et al., 1998; Pérez-Redondo et al., 1998) with two helix-turn-helix DNA-binding motifs at the amino and carboxyl ends of the protein. The *claR* gene is transcribed from a *tsp* site 155-nt upstream from the *claR* ATG start codon and, in certain mutants, from an adenine located 107-nt upstream of the start codon. Mutants disrupted in *claR* accumulate clavaminic acid but do not produce clavulanic acid and amplification of *claR* copy number results in a threefold increase of CA production (Paradkar et al., 1998; Pérez-Redondo et al., 1998). Increasing the copy number of either *claR*, *ccaR* and/or *ceaS2* genes has been used to obtain CA-overproducer strains (Kurt-Kizildoğan et al., 2017).

Regulatory cascades controlling clavulanic acid and cephamycin C biosynthesis

CcaR autoregulates its own expression as shown by binding of pure rCcaR protein to the *ccaR* upstream region. The CcaR-binding sites are duplicated/triplicated heptameric sequences characteristic of SARP-binding proteins that overlap with the *tsp1* −35 promoter region, as shown by DNAse footprinting (Fig. 1B). The regulation, at the transcription level of the cephamycin and CA biosynthesis gene expression, has been studied by transcriptomic analysis, RT-PCR studies and DNA-binding gel shift comparing the wild-type strain with *ccaR*-null mutants (Álvarez-Álvarez et al., 2014b; Kurt et al., 2013; Santamarta et al., 2011).

CcaR also binds the promoters of *cefD-cmcI, cefF* and *lat* in the cephamycin cluster activating the transcription of these genes. Indeed, as shown by qRT-PCR, disruption of *ccaR* results in a transcription decrease of all the genes encoding cephamycin biosynthetic enzymes. The stronger effect is exerted on *lat,* encoding the first step in the pathway, which is 1,700-fold downregulated in a *ccaR*-disrupted mutant; also downregulated are the *pcbAB* gene (300-fold) for the early steps of the pathway, *cefD* and *cefF* (300–400-fold) for the medium steps and *cmcI* (550-fold) for the late modifications steps (Kurt et al., 2013; Santamarta et al., 2011). Genes involved in β-lactam resistance (*pcbR, pcbA* or *bla*), or transport (*cmcT*) are not affected by the lack of CcaR. These results were confirmed by microarray analysis of a different *ccaR*-deleted mutant which showed an almost null expression for *lat, cmcI* and *cmcJ* (Álvarez-Álvarez et al., 2014b). Enhancing the amount of CcaR protein either using strong promoters or increasing *ccaR* copy number (Kurt et al., 2013) results in higher levels of cephamycin C production, as also occurs in industrial strains (Medema et al., 2011). A similar upregulation of the cephamycin biosynthesis genes was also observed by growing *S. clavuligerus* in conditions of nutrient rich as compared to nutrient-depleted medium (Pinilla et al., 2019).

CcaR acts on the clavulanic acid gene cluster by binding the *claR* promoter region as shown by gel-shift experiments (Kurt et al., 2013; Santamarta et al., 2011). *ccaR*-null mutants showed a 400-fold lower expression of *claR* and 3,000-fold downregulation of *ceaS2*, encoding the first enzyme of the CA pathway. Binding was not studied for other CA gene promoters, but the transcription of *cas2*, encoding the multifunctional clavaminate synthase, was 10,000-fold downregulated (Álvarez-Álvarez et al., 2014b; Santamarta et al., 2011).

Recombinant-CcaR protein binds to a triple heptameric sequence TCGATTC-8-TTCAAGA-14-TTCAGCG located upstream of the *claR* promoter as shown by EMSA assays and DNAse footprinting (Santamarta et al., 2011); qRT-PCR studies indicate that *claR* is expressed 190-fold less in a *ccaR*-disrupted mutant, confirming early Northern transcriptional analysis (Paradkar et al., 1998). Transcriptomic studies of a *claR*-deleted mutant showed that, in relation to the wild-type strain, the genes for the late steps (*oppA1, oppA2, car, cyp, orf12-13-14-16* and *gcsA*) were expressed at 1–8% level in relation to the wild-type strain while the genes for the early steps of the pathway were transcribed at 40–60% levels (Martínez-Burgo et al., 2015). In summary, ClaR is a CcaR-dependent activator that controls expression of genes for the late steps of CA biosynthesis but does not affect significantly expression of the early genes of the pathway (Paradkar et al., 1998) (Fig. 1A). The promoter sequences for ClaR-binding have not been yet reported.

Transcriptomic studies reveal additional global effects of CcaR and ClaR

Although CcaR appears to be a specific activator of cephamycin and clavulanic acid biosynthesis, transcriptomic analysis revealed that *ccaR*-deletion affects many additional genes for primary metabolism and secondary metabolite gene clusters (Álvarez-Álvarez et al., 2014b). Expression of genes related to energy production, carbon or nitrogen metabolism, phosphate metabolism, regulatory genes and genes for other physiological functions was affected at different degrees either in the exponential or stationary phase. Mutation of *ccaR* upregulated all the genes for holomycin biosynthesis an average value of 45-fold; downregulated an average of fourfold are all the arginine biosynthesis genes, and an average of 18-fold the genes for nitrogen metabolism (*amtB, glnB, glnA2, glnA3*) (Álvarez-Álvarez et al., 2013).

Similarly, transcriptomic studies of a *claR*-deleted mutant showed a weak upregulation of the cephamycin C genes at early times of the culture and, surprisingly, produced an upregulation of all the holomycin biosynthesis

genes (3- to 572-fold higher expression). The lack of ClaR affects the expression level of more than 100 genes for primary metabolism and regulators. Downregulated are also all genes of clusters encoding enzymes for two NRPS-type metabolites, the cucumene synthase, and an unknown product, respectively (SCLAV_p1334/_p1341, SCLAV_p1471/_p1483, SCLAV_p1407/_p1415 and SCLAV_p1508/_p1510); also downregulated is the gene *amfR* that controls aerial mycelium formation (Martínez-Burgo et al., 2015). However, no significant effects on expression of the clavam and paralogous gene clusters were found in the *claR*-deleted strain. These facts indicate that ClaR exerts a direct or indirect effect on many genes other than CA biosynthesis genes.

Additional mechanisms of regulation of ccaR expression

Expression of *ccaR* is controlled additionally by several master regulators (i.e., Brp, Are, AdpA) that bind to the intergenic *cmcH-ccaR* region. We will consider two different regulatory cascades, a 'proximal' regulatory cascade close to the UTR region and a 'distal' cascade acting near the *cmcH* gene.

Regulation of ccaR expression by Brp and AdpA

The proximal cascade starts with the well-conserved butyrolactone receptor protein Brp which was found by two research groups using different strategies (Kim et al., 2004; Santamarta et al., 2005). Brp is encoded by the pSCL4-located *brp* gene (Nishida et al., 2007) and, in the absence of butyrolactones, it controls its own expression, as shown by binding of pure Brp to an autoregulatory sequence (ARE$_{brp}$) upstream of *brp* (Santamarta et al., 2005).

Deletion of *brp* in S. *clavuligerus* results in an increase of clavulanic acid and cephamycin production (1.3- to 3-folds) in relation to the wild-type strain. Brp does not bind directly to the proximal UTR region of *ccaR* but its regulation is mediated through the AraC-type transcriptional regulator AdpA (López-García et al., 2010). Brp represses expression of *adpA* by binding to an ARE$_{adpA}$ sequence upstream of *adpA*. The AdpA protein is a strong activator affecting *ccaR* expression; transcriptional analysis reveals that removal of *adpA* in S. *clavuligerus* results in a decrease expression of *ccaR* (7-fold) and drastically reduces the transcription of *ceaS2/bls2* (50-fold) and *cas2* (100-fold) in the CA cluster resulting in very low production of clavulanic acid and cephamycin C. Conversely, amplification of *adpA* copy number in S. *clavuligerus* results in more than twofold production of cephamycin C and CA in relation to the control strain. This proximal cascade is highly similar to that involved in the control of the streptomycin regulator StrR (Tomono et al., 2005). In the UTR close to the *ccaR* gene we have found four putative AdpA-binding sequences (Ohnishi et al., 2005). Three of them are located 380- to 450-pb upstream of *tsp2* (Fig. 1B), one overlaps with the ATG codon of *ccaR* (Fig. 1B) and several putative, less conserved sites, are upstream of *tsp1*. Multiple AdpA-binding sites have been reported in the promoter of *sanR* encoding Streptomyces *ansochromogenes* activator of the nikkomycin cluster (Pan et al., 2009). In summary, all the available information indicates that the butyrolactone sensing system mediated by Brp controls the formation of AdpA which plays a major activator role in antibiotic production in S. *clavuligerus*.

In addition to the effect exerted through AdpA, Brp recognises an ARE$_{ccaR}$ sequence on the distant end of the intergenic *cmcH-ccaR* region. A regulatory protein of the IclR family, named AreB also binds to the ARE$_{ccaR}$ sequence, as shown by ARE$_{ccaR}$-affinity capture method (Santamarta et al., 2007). AreB is an autoregulatory protein that recognises sequences upstream overlapping the −35 site in its promoter controlling also the expression of the

leuCD genes, located tail to tail with the *areB* gene. The binding requires the presence of a small molecular weight ligand of still unknown structure, that may be leucine or a related branched chain amino acid (Santamarta et al., 2007; Yang et al., 2009). The significance of the Brp and AreB binding to this distal ARE$_{ccaR}$ sequence in cephamycin and clavulanic acid production is unclear since the ARE sequence is located almost 900-nt upstream of *tsp1*. Deletion of the *areB* gene produces a partial requirement of leucine for optimal growth but has no significant effect on biosynthesis of CA and cephamycin C.

Regulation by BldG

A regulatory protein that affects *ccaR* expression in a DNA-binding independent way is BldG, an anti-anti-sigma factor. Deletion of *bldG* results in mutants unable to differentiate and to produce cephamycin C and clavulanic acid due to lack of *ccaR* expression as shown by Northern analysis (Bignell et al., 2005). Complementation studies with the wild-type *bldG* allele resulted in the recovery of both β-lactam antibiotics formation. The *bldG* gene is located upstream of a gene encoding an anti-sigma factor. This organisation is identical to that of *Bacillus subtilis* in which the anti-sigma protein SpoIIAA binds the cognate sigma^F factor preventing its association to the core of the RNA polymerase and the transcription of the genes targeted by the RNApol-sigma^F. The sigma^F-SpoIIAA association is released by the binding of an unphosphorylated anti-anti sigma factor. In S. *clavuligerus* a similar mechanism of action of BldG with the anti-sigma factor encoded by the *bldG*-adjacent gene has not been experimentally demonstrated. The null *bldG* mutant lacks also production of the antifungal clavams, which are not CcaR-dependent; this suggests that the sigma factor controlled by BldG, binds the RNA polymerase and expresses both *ccaR* and the genes for clavams.

Regulation of clavulanic acid and cephamycin C biosynthesis by relA

The *relA* gene in actinobacteria and Gram-negative bacteria encodes a guanosine tetraphosphate synthetase that forms the highly phosphorylated nucleotide ppGpp. Initially the ppGpp molecule was studied as an intermediate of the stringent response; however, its role is more complex, affecting many genes involved in growth, differentiation and secondary metabolite biosynthesis (Ochi, 1990). The S. *clavuligerus relA* gene was cloned independently by two research groups and is located upstream of *apt*, encoding an adenosine phosphoribosyl transferase. Notably, the intergenic region *relA-apt* was reported to be 29 nt long by Jin et al. (2004) and 176 nt long by Gomez-Escribano et al. (2008). S1 mapping revealed that the *relA tsp* is 43-nt upstream of the TTG start codon suggesting that the S. *clavuligerus* strain studied by Jin et al. (2004) suffered a rearrangement in this region that explains the different behaviour of both strains. The deleted mutant S. *clavuligerus* Δ*relA* failed to produce aerial mycelium, spores or brown pigment in different solid media (Gomez-Escribano et al., 2008). Surprisingly, this mutant produced three- to fourfold more CA and 2.5-fold more cephamycin C than the parental strain. All these phenotypes were restored, and the antibiotics production decreased to wild-type levels, by complementing the Δ*relA* mutant with the wild-type *relA* allele (Gomez-Escribano et al., 2008). Expression of both *ccaR* and *claR* were higher in the mutant, mainly at the stationary phase that resulted in a longer period of antibiotics production. Particularly high was the expression of *ceaS2*, catalysing the first step of CA biosynthesis, and of *cefD* for the medium steps of cephamycin biosynthesis. The differences in *claR* expression at the stationary phase parallel the activation of an

additional transcription start point (*tsp2*), an adenine located 107-nt upstream of the ATG codon of *claR*. *tsp2* is poorly active, and probably repressed by ppGpp, in the wild-type strain. This suggests that ppGpp acts as a negative effector in cephamycin C/clavulanic acid production in *S. clavuligerus*.

Other regulators

In a different study the *pimM* gene, encoding the *Streptomyces natalensis* PAS-LuxR-type transcriptional regulator of the pimaricin gene cluster, was integrated in *S. clavuligerus* genome (Martinez-Burgo et al., 2019). The *pimM* gene, when expressed in *Streptomyces avermitilis, Streptomyces filipensis* or *Streptomyces nodosus* activates expression of filipin and amphotericin B gene clusters and in *Streptomyces albus* that of the polyketide/non-ribosomal peptide antimycin (Olano et al., 2014). Heterologous expression of *pimM* in *S. clavuligerus* resulted in downregulation of all the genes in clusters for a not identified terpene and a clavam-like *β*-lactam (SCLAV_5670/_5674 and SCLAV_p1508/_1510, respectively); it causes also the upregulation of the cluster for non-characterised polyketide antibiotic (SCLAV_p0509/_p0520) and for the clavulanic and cephamycin C gene cluster. All the genes for CA biosynthesis were upregulated (1.4- to 8-fold) and the CA production increased 10-folds in relation to the control strain, while genes for clavam biosynthesis were not affected. A similar effect was observed on all the genes for cephamycin C biosynthesis, with the exception of *pcd*, and cephamycin C production increased sevenfold. Overproduction of both antibiotics coincides with an upregulation expression of *ccaR* (11-fold) and *claR* (2-fold) genes. Pure PimM binds to the intergenic *ccaR-cmcH* region resulting in gel shift in EMSA, and a bioinformatic analysis revealed in the intergenic region a sequence similar to the PimM-binding sequence, 5′-GTCCGGAAATCCTCGG-3′, with Schneider nucleotide conservation values, R_i, of 8.6 and 9.9 for the direct and reverse strands (Fig. 1B). This suggests that a PimM-like activator might exist in *S. clavuligerus*.

Three genes, *orf21-orf22-orf23* (SCLAV_1477 to _1475), located in the CA gene cluster upstream of *gcaS,* have been studied as putative regulators of the cluster (Fu et al., 2019a; Song et al., 2009). *Orf21* encodes an RNA polymerase sigma[70]-family factor, and *orf22-orf23* (SCLAV_4176–4175) encode a two-component system (TCS) in which the sensor kinase *orf22* contains a histidine residue (His[283]) for autophosphorylation and *orf23* is the response regulator.

Transcription of the CA and cephamycin gene clusters, including *ccaR,* was not affected in the *orf23*-disrupted mutant*,* except for *lat* and *claR* transcripts, that were slightly downregulated. However, production of CA by the *orf22* and *orf23* mutants was decreased by 25% and 60%, while cephamycin C production was reduced by 40% and 60%, respectively. Only the *orf23*-null mutant showed delayed growth and poor sporulation (Song et al., 2009).

A double mutant, named *cagRS*, lacking both *orf22* and *orf23* has been obtained from the industrial strain *S. clavuligerus* F613-1 (Fu et al., 2019a). The *cagRS* mutant shows a strong downregulation of several genes for fatty acid degradation and a slight downregulation of glycerol metabolism genes. The mutation also resulted in downregulated expression of the arginine biosynthesis genes and of *oat2*, the CA cluster located gene encoding an ornithine aminotransferase. Recombinant CagR protein binds the *argC* and *argG* promoters as shown by EMSA gel shift, but the binding site or the relation with the ArgR repressor protein have not been studied. Expression of CA biosynthesis genes was not significantly affected in this mutant, although CagR was shown to bind the promoter of

claR and the double mutant *cagRS* produced 25–60% less CA. Integration of these results in the current knowledge obtained from the wild-type strain is not easy. The study of industrial strains is important for the producer company; however, these are heavily mutagenised strains that are grown in industrial complex culture medium; therefore, their regulation might be different from that of the wild-type strain and the results obtained in industrial strains should be always confirmed in parallel with the wild-type strain. In summary, the proteins encoded by *orf21, orf22, orf23* appear to have different effects on cell metabolism and may indirectly modulate the clavulanic acid and cephamycin C formation but should not be considered strictly regulators of the gene supercluster.

The effect on cephamycin production of a different TCS, named CepRS, (SCLAV_2959/2960), has been studied in the industrial strain *S. clavuligerus* F613-1 (Fu et al., 2019b). The mutant *S. clavuligerus cepRS,* deleted in *cepR* and *cepS* was found to produce normal levels of CA but only 30% of cephamycin C in relation to the parental strain. The CefR protein binds the intergenic *cefD-cmcI* region that controls genes encoding enzymes for the early, middle and late steps of the cephamycin pathway. The binding site appears to overlap with the three heptameric regions bound by CcaR, suggesting that CepR partially competes with CcaR, resulting in downregulation of the transcripts for the cephamycin biosynthesis genes. However, the *cefD-cmcI* region in strain F613-1 is slightly longer than in the ATCC 27064 strain and the binding site has not been clearly defined by footprinting.

Holomycin

Holomycin is a compound with dithiopyrrolone structure and yellow colour. Dithiopyrrolones are formed by two heterocyclic rings; one of them contains a disulphide bridge and the second has a nitrogen atom in the heterocycle (Fig. 2). The nitrogen-carrying heterocycle possesses an amino group, that is acetylated in holomycin, and propionylated in *N*-propionylholothin. Holomycin and related dithiopyrrolones are produced by Gram-negative bacteria and by actinomycetes, such as *Saccharothrix* species. More complex compounds containing the dithiopyrrolone chromophore (thiomarinols, xenorhabdins) are produced by enterobacteria that are mutualist or pathogens of invertebrates, and by marine bacteria (for reviews, see Li et al., 2014; Liras, 2014; Qin et al., 2013). *S. clavuligerus* IT1 was reported to produce a compound active against *Micrococcus luteus* that was not detectable in *S. clavuligerus* ATCC 27064 cultures; the compound was crystallised and identified as holomycin by ^{1}H-NMR and MS spectrum (Kenig & Reading, 1979). Later, holomycin was confirmed to be produced by a mutant defective in the *oppA2* gene. The yellow compound, is produced in increasing amounts by mutants blocked respectively in *car, claR, orf12, oppA2* and *cyp-fd,* while mutants blocked in the early steps of the CA pathway were holomycin non-producers (Fuente et al., 2002; Lorenzana et al., 2004).

The cluster of holomycin biosynthesis genes (Fig. S3A) was detected by bioinformatic analysis of the genes encoding the putative enzymes involved in holomycin biosynthesis (Huang et al., 2011; Li & Walsh, 2010) and heterologous expression of *hlmE* and *hlmA*. HlmE is a non-ribosomal peptide synthetase able to activate L-cysteine to form L-cys-AMP while HlmA is an acetyl transferase converting holothin into holomycin. Deletion of *hlmE* and of *hlmF,* encoding a lantibiotic-like decarboxylase, originated holomycin non-producer mutants (Li & Walsh, 2010). Independently, other research group observed *S. clavuligerus oppA2::aph* proteome intense protein spots corresponding to HlmD, HlmF and HlmG and
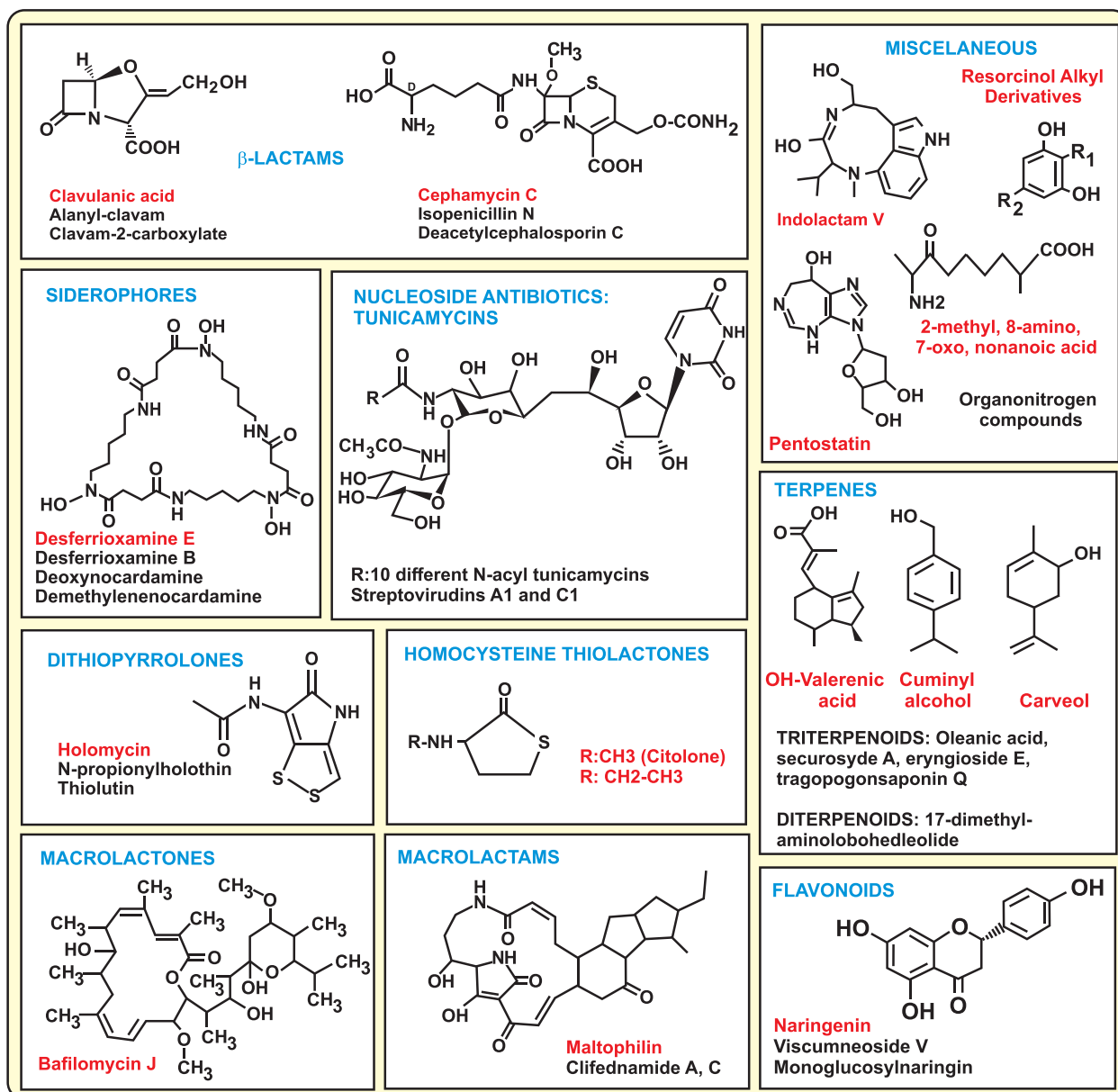
Fig. 2. Chemical compounds produced by *Streptomyces clavuligerus* as demonstrated by HPLC LC/MS analysis. When more than one compound of each type is produced, the structure shown corresponds to the compound labelled with red letters.

a high transcription of genes located adjacent to *hlmDFG* which was not observed in the wild-type strain; holomycin production in *S. coelicolor* by heterologous expression of the *hlm* genes confirmed that this region corresponds to the holomycin biosynthesis gene cluster (Robles-Reglero et al., 2013). The *hlm* cluster has been expressed with low efficiency in *S. albus* and *S. coelicolor* (Huang et al., 2011; Robles-Reglero et al., 2013). A biosynthetic pathway for holomycin (Fig. S3B) has been proposed after purification and studies of the recombinant proteins HlmA, HlmC, HlmD, HlmE, HlmF and HlmI, and after deletion of the *hlmF, hlmI* and *hlmE* genes (Li & Walsh, 2010, 2011). A key enzyme in the pathway is HlmI, an oxygen-dependent dithiol oxidase, that oxidises the disulphide bond forming the heterologous ring and produces holomycin from reduced holomycin, a metal-chelating compound (Chan et al., 2017). Mutants in *hlmI* show lower resistance to holomycin, although additional genes conferring holomycin resistance cannot be excluded. Proteomic studies show that overproduction of holomycin correlates with an intense protein spot corresponding to a thiosulphate sulphur transferase, RhlA. This protein is essential for holomycin overproduction, although the *rhlA* gene is not located in the *hlm* gene cluster (Nárdiz et al., 2011).

Transcriptional studies in the *oppA2*-deleted mutant *S. clavuligerus* Δ*oppA2::acc* showed a twofold average increased expression of all the *hlm* genes at three sampling times in relation to the wild-type strain. However, comparison with cultures of *S. clavuligerus* pSCL4⁻ indicated that the lack of plasmid pSCL4 affects the *hlm* genes expression more intensely (12.6-fold increase). This transcription deregulation of the pSCL⁻ mutant holomycin cluster is independent of the *oppA2* deletion and both effects are cumulative resulting in a 42.84-fold expression in a double mutant lacking *oppA2* and the pSCL4 plasmid (Álvarez-Álvarez et al., 2018). The effect was especially high on genes *hlmA* to *hlmI*, while less affected were genes *hlmK*, *hlmL* and *hlmM*, the last one encoding a LuxR-like transcriptional regulator.

The overproduction of holomycin by *S. clavuligerus* pSCL4⁻ might be explained if one of the genes located in pSCL4 encodes a repressor of the *hlm* gene cluster expression. In addition, pSCL4 contains several genes involved in sulphur, cysteine and methionine metabolism and their absence might channel sulphur-containing amino acids to produce dithiopyrrolones. However, the reasons for the overexpression of holomycin in clavulanic acid mutants blocked in the late, but not in the early steps of the CA pathway, is not understood.

Tunicamycin

Tunicamycins are a family of nucleoside antibiotics produced by several species of actinomycetes. They are bacterial cell wall inhibitors which target the translocase I involved in the first steps of peptidoglycan biosynthesis, affect the first step of protein glycosylation in eukaryotes, and also act as inhibitors of the palmitoyl transferase (Price & Tsvetanova, 2007). The tunicamycin structures are formed by a uracil molecule, *N*-acetylglucosamine and the rare sugar tunicamine (Fig. 2); members of the complex differ in the length of a lateral *N*-acyl chain (Tsvetanova & Price, 2001). Heterologous expression in *Streptomyces lividans* of a cosmid library of *Streptomyces chartreusis,* a tunicamycin producer, and HPLC analysis of the transformants culture broth revealed in an *S. lividans* transformant two novel peaks with antibiotic activity. LC/MS and MS/MS analysis and comparison with pure samples of tunicamycins identified the peaks as tunicamycin 15:1B and tunicamycin 16:1A (Chen et al., 2010). A biosynthetic pathway for tunicamycin has been proposed based on the characteristics of the *S. chartreusis tun* genes and in previous information (Chen et al., 2010; Tsvetanova & Price, 2001).

The *S. clavuligerus* IT1 strain reported to produce holomycin produces in addition a complex antibiotic, MM19290, formed by tunicamycins with *N*-acyl chains of 9–12 carbons (Kenig & Reading, 1979). Bioinformatic analysis of *S. clavuligerus* genome detected a cluster of genes (SCLAV_4275/_4287) almost identical in organisation to the tunicamycin cluster in *S. chartreusis* (Chen et al., 2010). This *tun* cluster is upregulated in *S. clavuligerus* pSCL4⁻ lacking the megaplasmid pSCL4, showing overexpression of all the genes an average value of 6- to 25-fold in relation to the wild-type strain. The more upexpressed genes were *tunA* and *tunB,* encoding enzymes for the early steps of the pathway (Álvarez-Álvarez et al., 2017). This result suggests that a gene located in pSCL4 encodes a regulator that controls negatively the tunicamycin biosynthesis genes.

Transformation of *S. clavuligerus* with the *pimM* gene (see above) affects also tunicamycin production. Expression of *pimM* did not modify significantly the transcription of the *tun* genes; however, the transformant strain, but not of the control strain, showed bioactivity against yeasts. The LC-MS metabolic profile of *S. clavuligerus::pimM* cell extracts showed notable differences in metabolites in relation to the control strain, and two peaks with antifungal activity were shown to correspond to tunicamycins. Tunicamycin production in the transformant was 5.3-fold higher than in the control strain and included tunicamycins with *N*-acyl chains of 11, 12 and 18 carbons as shown by MS quantification Interestingly, the higher antifungal activity (4- to 16-fold) corresponded to tunicamycin with longer acyl chains (i.e., 17:1) that were shown for the first time to be more active than those of shorter acyl chains (Martínez-Burgo et al., 2019). Since no differences in expression of the *tun* biosynthesis genes was correlated with *pimM* expression it was proposed that the overproduction of tunicamycin was due to the effect of PimM on genes that might supply additional precursors for tunicamycin formation, that is, for new and longer acyl lateral chains.

Streptovirudins, aminoglycosides identical to tunicamycins that contains dihydrouracil instead of uracil, have been described to be produced by ribosome engineered strains of *S. clavuligerus* (Shaikh et al., 2021). These authors suggest that the *tun* cluster encodes also the streptovirudins, although the gene responsible for the dihydrouracil formation is not present in the *tun* cluster.

Naringenin

Comparative HPLC analysis of broths from *S. clavuligerus* cultures grown in complex TSB and SA media revealed a peak in the former cultures that was not present in SA-grown cells. The compound was purified and analysed by LC-MS and ¹H-NMR. The substance was a flavonoid with a chemical structure corresponding to naringenin and differs from genistein in the lack of a double bond (Fig. 2). Naringenin is a typical plant compound not described previously to be produced naturally by microorganisms. The biosynthesis of naringenin in plants starts with the condensation of p-coumaroyl-CoA with three malonyl-CoA molecules by a chalcone synthase-type III. By comparison of genes/proteins in plants two clustered genes, *ncyP* and *ncs* (SCLAV_5491/5492), were located in *S. clavuligerus* genome (Álvarez-Álvarez et al., 2015). These genes encode, respectively, a cytochrome P450 oxygenase and a type III polyketide synthase with similarity to naringenin chalcone synthases. An additional gene, named *tal* (SCLAV_5457) was located 47-kb upstream of the cluster *ncs-ncyP. The tal* gene is similar to the *encP* gene of *Streptomyces maritimus,* which encodes a phenylalanine/tyrosine ammonia lyase (Tal) required for p-coumaroyl biosynthesis. Independent deletion of *S. clavuligerus ncs, ncyP* and *tal* genes resulted in strains unable to produce naringenin, and the production reverted to wild-type levels in each case by complementation with the deleted gene (Álvarez-Álvarez et al., 2015). The phenylalanine/tyrosine ammonia lyase protein has been purified to homogeneity; it has a prosthetic group methyl-idene-imidazol-5-one (MIO) giving the MIO characteristic spectrophotometric absorption; however, the Tal protein was very unstable which prevented enzymatic studies (Álvarez-Álvarez, personal communication). Supplementation of the cultures with fatty acids, that is, palmitic acid, and aromatic amino acids, as phenylalanine, improved naringenin production 3.7-fold. Under the optimal culture conditions studied *S. clavuligerus* ATCC 27064 produced 184 μg/ml naringenin. The fact that a natural compound is obtained from non-clustered genes, suggests that additional secondary metabolites might be encoded by *Streptomyces* species by distantly located (unlinked) genes.

Three flavonoid glycosides, including viscimneoside V and monoglucosyl-naringin, are produced by *S. clavuligerus* mutants (Shaikh et al., 2021). Adjacent to *ncyP* and *ncs* there are genes for a transketolase (SCLAV_5490) and a transglycosidase (SCLAV_5493); therefore, these authors suggest that these flavonoids are produced by the same gene cluster than naringenin.

Metabolomics

We have described above the compounds produced by *S. clavuligerus* from which enzymes and pathways are known, although in some of them, as is the case of tunicamycin, the knowledge partially relies on studies in other microorganisms.

Up to 58 secondary metabolite gene clusters have been described in *S. clavuligerus* genome (Hwang et al., 2019). Most of them are cryptic or silent clusters that have not been characterised, and

the compounds derived from many of these clusters are still not known. Considering that, in any case, more than one compound might be encoded by every gene cluster the number of secondary metabolites potentially produced by *S. clavuligerus* is well over one hundred. Several strategies have been followed to characterise these secondary metabolites and the clusters responsible for their formation. This knowledge is important in order to delete the active clusters in industrial strains as a way to improve clavulanic acid production.

Clusters Characterised by the Similarity of Their Genes/Proteins to Those Present in Other Microorganisms

Tacrolimus. Tacrolimus (FK506) is a macrolide compound with inmunosupressant activity produced by *Streptomyces tsukubaensis* (Ordóñez-Robles et al., 2018). The cluster *fkb* for tacrolimus biosynthesis in *S. tsukubaensis* is formed by 16 ORFs and contains 3 genes for polyketide synthetases (*fkbA* to *fkbC*) and two genes for regulatory proteins (*fkbN* and *fkbR*). Mo et al. (2009) described the production of tacrolimus by *S. clavuligerus* CKD1119. Later Park et al. (2009) using LC/MS analysis reported the presence of tacrolimus and eight intermediates of the biosynthesis pathway in *S. clavuligerus* KCTC10561BP cultures.

In *S. clavuligerus* ATCC 27064 genome only genes SCLAV_0006 to _0010 have a similar organisation to the tacrolimus gene cluster as they encode PKSs with 45–50% identity to FkbA to FkbC. However, the proteins encoded by the surrounding genes have low identity to the Fkb proteins and the cluster lacks genes homologous to *fkbO* and *fkbL*. Moreover, the cluster SCLAV_0006 to _0017 has been found recently to be identical to that of the bafilomycin cluster of *Streptomyces lohii* (Zhang et al., 2013) and has been proposed that this cluster is responsible for bafilomycin production in *S. clavuligerus* (Shaikh et al., 2021). Therefore, *S. clavuligerus* ATCC 27064 does not appear to have a cluster for tacrolimus biosynthesis and was never reported to produce tacrolimus. It would be of interest to compare the genomes of the wild-type strain and the reported tacrolimus producers *S. clavuligerus* strains to clarify whether these strains obtained the tacrolimus cluster by horizontal transfer.

Moenomycins. Moenomycins are phosphoglycolipids acting on the glycosyltransferases involved in bacterial cell wall biosynthesis. These compounds are produced by *Streptomyces ghanaensis* (Ostash et al., 2009) while teichomycins, belonging to the moenomycin family, are produced by *Actinoplanes teichomyceticus*. A cluster of genes located in *S. clavuligerus* plasmid pSCL4 (SCLAV_p1274/_p1290) is similar to the *moe* and *tchm* clusters and encode proteins 47–82% identical, what suggests that this is a moenomycin gene cluster. Moenomycins could not be detected in fermentations of *S. clavuligerus* ATCC 27064 suggesting that this is a silent cluster under the studied conditions (Álvarez-Álvarez et al., 2017).

Compounds Characterised by HPLC-LC/Mass Spectrometry Analysis of Culture Broths

Comparison by LC/MS-MS analysis of *S. clavuligerus* ATCC 27064 culture broth and that of different mutants allowed the characterisation of several compounds (Álvarez-Álvarez et al., 2017; AbuSara et al., 2019; Martínez-Burgo et al., 2019).

The pSCL4-less mutant produces holomycin and *N*-propionylholothin, which are not detectable in *S. clavuligerus* ATCC 27064, in agreement with the upregulation of the holomycin

biosynthesis genes in this mutant (Álvarez-Álvarez et al., 2017). The molecular mass of *N*-propionylholothin and thiolutin are identical but only *N*-propionylholothin has been detected by MS analysis to be produced by *S. clavuligerus* (Okamura et al., 1977). Two thiolactone compounds carrying sulphur in the molecule, *N*-acetyl and *N*-propionyl homocysteine, were also detected in the pSCL4-less strain. Nocardamine (desferrioxamine E) was the main desferrioxamine in both strains although small amounts of demethylenocardamine and deoxynocardamine were also present in the wild-type strain broth. The wild-type strain but not the pSCL4-less mutant produced resorcinol-derived compounds and methyl-7-oxononanoic acid (Álvarez-Álvarez et al., 2017) (Fig. 2).

A mixture of four tunicamycins, not completely characterised, was reported to be produced by *S. clavuligerus* (Kenig & Reading, 1979). Recently, the heterologous expression in *S. clavuligerus* of *S. natalensis pimM* gene (see Other regulators section) showed that the transformant produced eight members of the tunicamycin family with acyl chains of 11–18 carbons. They include two previously unreported compounds with acyl chains of 11 and 18 carbon atoms (Martínez-Burgo et al., 2019).

AbuSara et al. (2019) in a comparative study of the metabolome of *S. clavuligerus* confirmed the formation of desferrioxamine E (nocardamine), naringenin, holomycin and the tunicamycins by *S. clavuligerus*. In addition, using LC-MS and MS-MS these authors described the presence of several new compounds, as the terpenes carveol, cuminyl alcohol and hydroxyvalerenic acid, the linear siderophore desferrioxamine B, indolactamV and the nucleoside pentostatin (Fig. 2). In addition, this research group performed engineering of *S. clavuligerus* ribosomes overexpressing the *rpsL* for a ribosomal protein, the *frr* gene, for a recycling factor and obtaining by direct mutagenesis different mutants of *rpsL*. LC-MS/MS analysis of culture broth in these *S. clavuligerus* mutants revealed the presence of 28 new metabolites. They include nucleoside antibiotics as streptovirudins A1 and C1; macrolactone antibiotics as bafilomycin J; macrolactams as maltophilin and clifednamides A or B; flavonoids as viscumneoside V and monoglucosylnaringin; 10 different triterpenoids including oleanic acid and the saponins secursyde A, eryngioside E and tragopogonsaponin Q, three different diterpenoids, the hopanoids glochidone and 23-OH-hopen-1-one, and several organonitrogen compounds (Shaikh et al., 2021). Gene clusters for some of these compounds were suggested by the authors, but they still require confirmation.

Characterisation of *Streptomyces clavuligerus* Terpene Synthases by Heterologous Expression

Terpenoids used in the cosmetic and food industries as flavour and odour constituents have been isolated mostly from higher plants, and are rarely produced by microorganisms. Terpenoids derive from isopentenyl diphosphate (C5) and dimethylallyl diphosphate (C5), and by successive condensations form geranyl diphosphate (GPP, C10), farnesyl diphosphate (FPP, C15) and geranylgeranyl diphosphate (GGPP, C20). Cyclisation of these intermediates by terpene synthases/cyclases and further modifications result in the formation of different monoterpenes (C10), sesquiterpenes (C15) and diterpenes (C20).

Terpenoid platform vectors, containing inducible genes for the mevalonate pathway and/or for the precursors FPP, GPP or GGPP (*fps, gps* or *ggs* genes) expressed from strong promoters have been constructed in modified *Escherichia coli* strains (Hu et al., 2011; Yamada et al., 2016; Karuppiah et al., 2017) to produce terpenes.

Table 2. Terpenes Characterised in Cultures of Heterologously Expressed Terpene Synthases of *Streptomyces clavuligerus*

S. clavuligerus gene	Protein name	Substrate	Product	Reference
SCLAV_p0328		FPP	(-)-δ-cadinene	Hu et al., 2011
SCLAV_p0068		FPP	(+)-T-muurolol	Hu et al., 2011
SCLAV_p0068/SCLAV_00067			(-)-drimenol	Yamada et al., 2015a
SCLAV_p0982	CinS	GPP	1,8 cineole, β-pinene, camphene	Nakano et al., 2011a; Yamada et al., 2015b.
SCLAV_p1185	LinS	FPP y GPP	Linalool and nerolidol	Nakano et al., 2011b
SCLAV_p0765		GPP	Hydropyrenol, hydropyrene, isoelisabethatriene B	Yamada et al., 2015a,b
SCLAV_p1169		GPP	Clavutrienes A and B, prenylgermacrene B, lobophytumin C	Yamada et al., 2015b
SCLAV _1407		FPP	(5S, 7S, 10R, 11S) cucumene (iso-hirsutene)	Yamada et al., 2015b Chow et al., 2015
SCLAV_p0490/_p0491		GGPP	Labda 7,13(16), 14 triene	Yamada et al., 2016

More than 20 genes for terpene synthases have been found in *S. clavuligerus* genome (Hu et al., 2011). Most *S. clavuligerus* terpene synthase genes are located in plasmid pSCL4 and encode terpene synthases type I which contain the characteristic Mg^{2+}-binding amino acid motifs (Yamada et al., 2016). About half of these genes have been expressed in *E. coli* and GC-MS/NMR of the transformants broth detected the production of monoterpene, sesquiterpene or diterpenes (Table 2).

Two genes, SCLAV_p0328 and SCLAV_p0068, encoding terpene synthases, flanked by genes for methyltransferases and cytochrome P450, probably involved in later modifications of the produced terpene, have been expressed in *E. coli*. The recombinant proteins incubated with FPP generated, respectively, a sesquiterpene ($C_{15}H_{24}$), a sesquiterpene alcohol ($C_{15}H_{26}O$) identified as (−)-δ-cadinene, and (+)-T-muurolol (Hu et al., 2011). In addition, the SCLAV-p0068 gene, when coexpressed with SCLAV_p0067, encoding a cytochrome P_{450}, forms (−)-drimenol (Yamada et al., 2015a,b) (Fig. S4).

In parallel to the previous work, Nakano et al. (2011a) described that SCLAV_p0982 (*cinS*) encodes a type I monoterpene synthase, which uses only GPP as substrate and catalyses the synthesis of 1,8 cineole (eucalyptol), an essential oil used in the pharmaceutical and cosmetic industries, which is extracted normally from *Eucalyptus polybractea*. This gene has been described also as producer of β-pinene and camphene (Yamada et al., 2015b).

A different gene, SCLAV_p1185 (*linS*), encodes the linalool synthase. LinS uses FPP to produce nerolidol, and GPP to produce linalool (Nakano et al., 2011a, 2011b) as confirmed by GC-MS and comparison with pure compounds, but is unable to use GGPP. Linalool is a product used as fragrance in hygiene products; improved expression of the linalool synthase and strains with a better GPP assimilation resulted in a linalool yield of 1,027 mg/l in fed-batch cultures (Wang et al., 2020). However neither linalool, nerolidol or 1,8 cineole have been ever detected in *S. clavuligerus* cultures.

Labda 7,13(16), 14 triene, is formed in *E. coli* as product of the genes SCLAV_p0490/SCLAV_p0491. This is a novel triene (Fig. S4) which was not observed in *S. clavuligerus* cultures grown under different conditions. The cluster of genes SCLAV_p0484 to SCLAV_p0491, located in plasmid pSCL4, carry in addition to genes for terpene synthases, genes for a transcriptional regulator, a geranyl geranyl phosphate synthase (GGPPS) and a transport system (Yamada et al., 2016). Four different labda-type terpenes have been obtained by combined expression of SCLAV_p0490/_p0491

with genes for terpene cyclases of *Streptomyces cyslabdanicus* and *Streptomyces anulatus* (Yamada et al., 2016).

The *S. clavuligerus* cucumene synthase and terpene synthases LinS and CinS, have been crystalyzed (Blank et al., 2018; Karuppiah et al., 2017). These proteins are relatively unique since they lack the N-terminal alpha barrel domain present in the corresponding plant terpene synthases. The enzymatic reaction of several *S. clavuligerus* terpene synthases have been thoroughly analysed (Chow et al., 2015; Rinkel et al., 2016) since the modification of the key amino acid residues required for catalytic activity might allow to obtain modified monoterpenoids.

From all these results it can be concluded that *S. clavuligerus* has the ability to produce terpenes as shown by the detection of carveol, cumminyl alcohol or hydroxyvalerenic acid in culture broths (AbuSara et al., 2019); however, the wealth of terpenes obtained in heterologous expression platforms has never been observed in *S. clavuligerus* cultures even although a variety of culture conditions have been tested (Yamada et al., 2015a). In fact, although a gene (SCLAV_p0159) for geosmin biosynthesis, a sesquiterpene responsible for the characteristic moist soil odour is present in the genome, this compound has never been detected in *S. clavuligerus* cultures (Yamada et al., 2015a).

The genes for terpene synthases are transcribed in *S. clavuligerus* as shown by downregulation of their expression in mutants lacking *claR* or pSCL4 (Álvarez-Álvarez et al., 2014a; Hwang et al., 2019; Martínez-Burgo et al., 2015), that is, they are not silent genes. The limited production of terpenes by *S. clavuligerus* cultures might be due to poor supply of precursors.

Conclusions

The advances in sequencing technology provides a high-coverage *S. clavuligerus* genome and provides an explanation for the instability found in plasmids and chromosome arms in different isolates of this species. The complexity of plasmid–chromosome relationships makes essential the confirmation of pSCL4 copy number and subsequent chromosome stability in every study. The genome sequence facilitates transcriptomic studies which still require a large amount of experimental research to connect the observations obtained by microarray analysis with the physiology and behaviour of *S. clavuligerus* in the different investigated conditions, especially in relation to primary metabolism.

Genomic studies provide also a wide view of the large number of clusters for secondary metabolism present in *S. clavuligerus*

genome. Metabolomic studies, heterologous expression and comparison with other genomes permit to determine the compound encoded by different secondary metabolite clusters, but confirmation through gene disruption and biological and chemical analysis of the mutants is needed in most cases. In conclusion a new era is open to study the genetics and physiology of S. *clavuligerus* in order to convert this strain in an instrument for the efficient production of new compounds of industrial interest. These multi-omic studies open the way for the utilisation of S. *clavuligerus* has a platform for industrial production of secondary metabolites in addition to clavulanic acid.

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Supplementary Material

Supplementary material is available online at *JIMB* (*www.academic.oup.com/jimb*).

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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